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In vitro studies on adenomatosis of the colon and rectum[1]

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SUMMARY Cell culture studies on adenomatosis of the colon and rectum (ACR) suggested that the clinical phenotype, colonic adenomas which become malignant recognised as a single clinical entity, may not be entirely the result of a single dominant mutation. Of the dermal cultures containing both epithelioid and fibroblastic cells established from six affected subjects from six unrelated ACR families, four showed increased tetraploidy and two did not. Of similar cultures established from four affected subjects from families with ACR associated with sebaceous cysts in consecutive generations, three did and one did not have increased tetraploidy.

Irrespective of the *in vivo* relationship of increased tetraploidy to colonic cancer, the cultures from seven ACR patients had populations of tetraploid cells, at least *in vitro*, with chromosome instability. Such a difference in expression of the ACR genotype *in vitro* suggested genetic heterogeneity within this clinically defined group.

Hereditary adenomatosis of the colon and rectum (ACR) is characterised by multiple adenomatous colonic polyps which show malignant transformation (Dukes, 1952). Although there are a number of hereditary polyposes of the gastrointestinal tract, ACR has been considered to represent a distinct clinical entity inherited through a single dominant gene (Cockayne, 1927).

The concept of a single polyposis gene for ACR has been accepted on the basis of family pedigrees and the location of lesions solely in the gastrointestinal tract, in spite of the great variation observed within this clinical syndrome (Dukes, 1952; Reed and Neel, 1955; Veale, 1965; Alm and Licznerski, 1973).

It now appears that ACR may be studied *in vitro* (Danes, 1976a, b). Increased tetraploidy has previously been reported to occur in epithelial-containing cultures from colonic mucosa and not from dermis derived from patients with ACR (Danes, 1978). Increased tetraploidy was not observed in all cultured cells with the ACR gene, but only in colonic mucosa which, although appearing normal *in vivo*, was known from clinical phenotypes and family histories to undergo malignant transformation *in vivo*.

It is proposed that if all ACR cases diagnosed on clinical criteria were the result of a single dominant gene, increased tetraploidy would not be observed in epithelial-containing dermal cultures, as malignant lesions *in vivo* are not observed in the skin. To test this hypothesis the occurrence of tetraploidy was studied in dermal cultures from ten affected subjects from ten unrelated families with ACR in consecutive generations (Alm, 1974) and ten normal subjects not at risk (age and sex matched).

Subjects and methods

Ten affected members (5 male, 5 female, aged 20 to 59 years) from ten unrelated families with ACR (six with colonic polyps only, four who also had sebaceous cysts) have been studied. Before this, one of us (TA) established the diagnosis in each affected member studied and the clinical phenotypes within each family on the basis of clinical data and family pedigrees derived from medical records and parish registers (Alm, 1974).

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For eight of the ten affected subjects studied, (patients 1 to 8, Table 1), the adenomatous colonic lesions (number of adenomas over 100 and their distribution over the entire bowel with the highest density in the rectum, sigmoid, and descending colon) were as expected to be found in ACR (Lockart-Mummery and Dukes, 1939; Dukes, 1952; Alm and Licznerski, 1973).

Patient 9 had multiple adenomas of sufficient number to be considered to be adenomatosis, but at the age of 59 had required only segmental resection which is unusual in ACR. In this family the patient's mother, maternal aunt, sister, son, and nephew all had ACR resulting either in early death from colonic cancer or in preventive surgery (ileostomy or ileorectal anastomosis) early in the second decade of life.

The tenth patient (Table 1) had only a single polyp at the age of 32 (removed by polypectomy) and had normal mucosa for the next ten years (at time of study). In this family of 12 affected members in three generations, a single family member, a paternal cousin of patient 10, had adenomatosis involving the entire colon so the family was considered to have ACR. Six affected subjects in two generations had colonic cancers limited to specific areas (caecum, transverse colon, or rectum); in two other members the cancer was located solely in the oesophagus.

The four affected (patients 5 to 8, Table 1) with sebaceous cysts in association with ACR were members of families with this association in consecutive generations. The colonic pathology (adenomatous polyps carpeting the colon) in these patients was indistinguishable from that found in the affected subjects without cysts or a family history of them (patients 1 to 4, Table 1).

Skin biopsies were obtained from each affected subject studied and from controls (age and sex matched). A coded number was assigned to each biopsy, which was the only identification used until the studies on tetraploidy had been completed. Skin cultures were established from these split-thickness biopsies by standard culture methods (Danes and Bearn, 1969). Cultures were grown in Falcon plastic petri dishes in Eagle's MEM with 20% (vol) newborn calf serum in an atmosphere of 5% CO_2 in air. To enhance the migration of epithelial cells from the dermis, a minimum amount of medium (approximately 1 ml) was used in the first culture weeks (just enough to cover the biopsy and floor of the culture dish). The pH of the medium was kept between 7·0 and 7·4 during the culture period. After two culture weeks the initial explant culture was examined microscopically to verify that both sheets of epithelial cells, presumably from the epidermis and fibroblasts from the dermis, were present in the migration zone surrounding the explant before trypsinisation to obtain a cell line. The cells were grown in culture

Table 1 *Clinical and cell culture phenotypes of 10 patients with adenomatosis of the colon and rectum and their age-sex matched normal controls*

Patient No	*Family No**	*Ident. No within pedigree**	*Sex*	*Age (y) at DX*	*Surgical Rx*		*Age (y) at study*	*Phenotypes*			*Organ sites of other tumours in family**
					Type	*Age (y)*		*Culture*		*Clinical*	
								% tetraploidy†		*Colonic lesions, polyps*	
								Patient	*Matched normal*		
1	1	IV.18	M	30	IRA	30	51	14	2	Carpeted	Stomach (1) Bladder (1) Cerebrum (1)
2	46	IV. 8	F	12	IRA	20	20	13	3	Carpeted	
3	114	II.3 P	M	24	TC	25	46	18	0	Carpeted	
4	130	P	M	27	IRA	29	35	4	4	Carpeted	
5	5	III.2	M	32	IRA	35	52	10	2	Carpeted	
6	18	IV.1 P	F	27	IRA	38	44	11	1	Carpeted, 'slow growing'	Gastric
7	54	III.2	F	38	SR IRA TC	35 39 40	49	8	3	Carpeted	
8	66	IV.19	F	35	IRA	35	47	3	0	Carpeted	Hypopharyngeal (1) Nasopharyngeal (1)
9	19	III.1 P	F	21	SR SR	34 39	59	3	0	Small adenomas (+ gastric)	
10	67	III.6	M	32	PY	32	42	10	4	Single	Oesophagus (2) Uterus (1)

*Data from pedigrees of families in the Appendix of pedigrees and tables in Alm, 1974.
P, proband; IRA, colectomy and ileorectal anastomosis; PY, polypectomy; SR, segmental resection of colon; TC, total proctocolectomy with ileostomy.
†Based on number of metaphases showing tetraploidy divided by total number of metaphases scored blind on slides having at least 100 divisions per slide.

from 12 to 20 weeks (5 to 8 subcultures by trypsinisation) before mitotic activity was studied.

Three observations were made to ascertain that epithelial cells were present in the skin lines to be studied for the occurrence of tetraploidy. (1) At trypsinisation each culture was checked microscopically to avoid cell selection. Each line was assayed for (2) resistance to dodecyl-sulphate, an index of the presence of cornified cellular envelopes seen in keratinocytes and squames (Sun and Green, 1977), and (3) keratin (Young, 1963).

For chromosome preparations on the sublines, the cells were trypsinised into suspension and approximately 10^4 cells were suspended in 5 ml of medium without antibiotics, inoculated into small Falcon petri dishes, and incubated in an atmosphere of 5% CO_2 in air. After 48 hours, when a burst of mitoses was observed, chromosome preparations were made with the addition of colchicine and stained in aceto-orcein.

Two slides of each preparation were made on two different cultures of each subline examined. Mitoses on the entire slide were examined, as it was found that bias could be introduced if only part of a slide was studied. As it was considered that a slide must contain 100 mitoses to reflect the mitotic activity of a subline, slides with less than 100 mitoses were not included in this study.

The occurrence (%) of tetraploidy in a culture was expressed as the number of metaphases showing tetraploidy divided by the total number of metaphases counted.

Results

A cell line established from a dermal biopsy was studied only if the cell population contained epithelial cells. Their presence was determined by (1) sheets of epithelial cells in the primary explant culture which were included in the first subculture by trypsinisation; (2) dodecyl-sulphate resistant cells; and (3) cellular keratin by chemical analysis in subcultures.

The occurrence of tetraploidy scored blind in sublines derived from the same biopsy showed little variation (Table 2). Sublines derived from biopsies obtained at least a year apart from a patient had a similar percentage of tetraploid mitoses (Table 2).

The cultures established from the normal subjects (age and sex matched) not at risk for ACR showed 0 to 4% tetraploidy (Table 1). The percentage of cells showing tetraploidy was approximately the same in all the sublines established from a single biopsy.

For the four patients (No 1 to 4, Table 1) with ACR, *in vitro* tetraploidy was increased in three (13, 14, and 18%) and not in one (4%). For the four patients (No 5 to 8, Table 1) with ACR and cysts, three showed increased tetraploidy (8, 10, and 11%) and one did not (3%).

The cultures from patient 9 (Table 1) with a number of small adenomas did not have increased tetraploidy (3%), whereas those from patient 10 (Table 1) with a single adenoma did (10%).

Discussion

In his Hunterian lecture in 1952, Dukes defined ACR as 'a hereditary disease characterised by the development within the colon and rectum of large numbers of adenomatous tumours', and discussed in detail the mode of inheritance (autosomal dominant first postulated by Cockayne in 1927) and the ultimate value of family studies.

Since then, family studies have been done by Reed and Neel (1955), Veale (1965), and Alm and Liczner-ski (1973) to clarify both the clinical expression and

Table 2 *Percentage of dividing cells showing tetraploidy in sublines derived from separate skin biopsies taken from each of 3 patients (5, 6, 8, Table 1) over a 2-year period*

Patient No	*Year biopsy taken and culture line established*	*TN**	*Percentage of dividing cells showing tetraploidy†*				
			Sublines established from primary explant culture				
			1	*2*	*3*	*4*	*5*
5	1976	6	9·7	10·5	9·4	9·8	11·0
		8	10·4	10·7	9·0	10·8	9·7
	1977	4	10·4	11·0	11·5	10·2	12·0
		9	12·6	12·0	14·5	11·4	10·4
6	1976	7	11·2	11·4	18·0	11·0	12·8
		8	10·5	14·6	10·4	15·8	17·2
	1978	5	14·0	12·6	13·8	18·0	15·8
		7	14·8	11·8	15·4	10·4	15·0
8	1977	4	3·0	2·8	2·4	1·8	2·9
		6	3·8	1·2	2·8	1·0	2·0
	1978	4	1·6	1·5	4·2	3·3	1·4
		9	1·4	1·7	3·0	3·6	1·0

*TN, trypsinisation No after sublines established at second subculture of primary explant culture.
†Based on No of metaphases showing tetraploidy divided by the total of No metaphases scored blind on slides having at least 100 divisions.

genetics within and between such families. Their studies verified Dukes's definition of ACR, with the average age of colonic symptoms at 20 years and the diagnosis of colonic cancer 15 years later.

Although colonic adenomas predisposing to cancer were found to be the common feature in all such ACR families, each investigator (Dukes, 1952; Reed and Neel, 1955; Veale, 1965; Alm and Licznerski, 1973) commented on the variation observed in clinical expression (number and distribution of such adenomas, age of onset) between and even within families. Such variability suggested that all ACR might not be the result of one single dominant gene mutation, the polyposis gene.

As there are no symptoms specific to ACR, and from a practical viewpoint proctosigmoidoscopy would not be feasible in the general population, a specific cell marker is needed to identify subjects who have inherited the genotype for ACR, who should receive periodic colonic examinations and eventually, when required, preventive colonic surgery.

In the present study (Table 1) the occurrence of tetraploidy in cultures established from skin biopsies from the ten subjects not at risk for ACR, age and sex matched with one of the ten ACR patients studied, was low (0 to 4%). Age and sex of the donor of a skin biopsy did not influence the occurrence of *in vitro* tetraploidy (Table 1) (Danes, 1975; Danes and Krush, 1977). Previous research (Danes, 1975, 1976a, b), as well as the present study (Table 2), has shown that the occurrence of tetraploidy in dermal cultures is similar in sublines derived from the same biopsy or from multiple biopsies from the same subject.

In a previous study increased *in vitro* tetraploidy occurred in dermal cultures from patients with Gardner's syndrome (defined as ACR with extracolonic lesions: osteomas, fibromas, and epidermoid cysts) but not in those without such extracolonic lesions (Danes, 1978). Therefore, it would have been predicted that the cultures derived from the ACR patients in the present study would not have shown increased *in vitro* tetraploidy. However, cultures from four of the six patients with ACR and three of the four with ACR with associated cysts showed increased tetraploidy (Table 1).

There are at least four possible explanations for increased tetraploidy being observed in dermal cultures derived from some but not all patients with ACR (Table 1).

As the increased incidence of *in vitro* tetraploidy in a mixed cell population has been shown (Danes, 1976b, 1978) to depend on the presence of an epithelioid cell population, the absence of increased tetraploidy might reflect a low number of this cell type in the sample on which mitotic activity was determined. The original observation (Danes, 1975) on the absence of increased *in vitro* tetraploidy in ACR was made before knowing that increased *in vitro* tetraploidy was tissue and cell type specific. In the present study only cultures which were shown by chemical analyses (cellular resistance to dodecyl-sulphate and intracellular keratin) to contain epithelial cells were included. There appeared to be no striking differences in the cell types in the cell populations from biopsies derived from normal subjects and those with ACR.

It was found that if the primary explant culture was just covered, rather than submerged, in medium, the sheet of epithelioid cells migrated further before losing contact with the culture floor, allowing for a greater epithelioid cell population and a reduction in the number of fibroblast cells. Such a procedure enhanced the proportion of epithelioid cells in the culture at the time of first trypsinisation. It was apparent that cultures of epithelial cells only were needed. Until then, it was not possible to establish definitely that all tetraploidy occurred in epithelial cells; such tissue specificity has been shown to be characteristic of hereditary tumours (Knudson *et al.*, 1973). However, it may be necessary to have fibroblastic cells present for epithelium to be maintained and grown in culture (Rheinwald and Green, 1975).

As increased tetraploidy has been shown to be a cell culture phenomenon and not to occur, at least in skin, *in vivo* (that is, to be present or occurring in the tissue biopsy before culture), a second possibility was that this mitotic abnormality could be secondary to *in vitro* agents (viral or chemical). Such a possibility has been virtually eliminated by obtaining the same results from cultures established from repeat biopsies from the same normal and affected subjects taken 6 months to 2 years later (Table 2). Such repeat cultures were grown in a different laboratory using a different source of medium. However, the introduction of viral contamination in some primary biopsies has not been ruled out.

The third possibility was that the clinical phenotype, colonic polyps, in these ten families was not the result of the same mutation, this genetic difference not being detected *in vivo* but rather *in vitro*.

It has been proposed that the colorectal cancer syndromes with polyposis are distinct genetic entities. The families studied (Table 1) represent subgroups: adenomatosis of the colon and rectum (classical ACR, patients 1 to 4); ACR with associated cysts (patients 5 to 8); and single polyps (patient 10). Patient 9 was a member of a classical ACR family but he had too many polyps to be included in the category of multiple polyps and a benign course. Although not proved, the assumption has been that

each subgroup represented different dominant mutations rather than variable expressivity (Veale, 1965; McConnell, 1966; Alm and Licznerski, 1973).

Veale (1965) suggested that linkage studies could provide tentative evidence for either the same or different loci being involved in ACR with and without cysts. In the present study the dermal cultures established from the ACR patients with and without cysts could not be divided into two subgroups on the basis of the occurrence of tetraploidy (Table 1). No experimental *in vitro* evidence was obtained to clarify whether the clinical phenotype of ACR with cysts was the result of a different mutation at the same or different locus, or whether the colonic lesions resulted from the polyposis gene and the cysts from another gene, presumably linked, or at least on the same chromosome.

In the present study only the proband in each of the ten families was studied (Table 1). In an *in vitro* study (Danes and Krush, 1977) on 137 members of six families with Gardner's syndrome, the cultures from all 28 affected members showed increased tetraploidy. It was possible to trace the inheritance of the Gardner gene through consecutive generations in all six families with the Gardner syndrome studied. Thus, determination of the occurrence of tetraploidy in other affected members in each of the ten families in the present study (Table 1) would help to determine whether such a difference in expression (presence or absence of increased *in vitro* tetraploidy) of the ACR genotype *in vitro* was the result of genetic heterogeneity.

The fourth possibility was that increased occurrence of tetraploidy was not the result of the polyposis gene, but of a modifying gene which is allelic on the homologous chromosome, or non-allelic on the same or different chromosome. The possible role of modifying genes in the expression of the polyposis gene was proposed by Veale (1965) and discussed by McConnell (1966). The occurrence of tetraploidy in cultures established from ACR families including all matings in consecutive generations should provide such evidence for the possible existence of such a modifying gene/genes.

The differences in the presence of *in vitro* tetraploidy in ACR families may eventually be relevant to the *in vivo* variability in clinical expression remarked on by the investigators involved in family studies (Dukes, 1952; Reed and Neel, 1955; Veale, 1965; Alm and Licznerski, 1973).

Boveri (1914) postulated that chromosomal aberrations were important in the aetiology of malignancy. Tetraploidy, assumed to be the result of endoreduplication, in the cultured cell produces such chromosomal instability. If such a chromosomal abnormality occurred solely in cells which would eventually be transformed into cancer cells, then this phenotypic abnormality might be a reflection of a first or subsequent step in the multistep model for cancer (Nordling, 1953; Knudson *et al.*, 1973). Increased tetraploidy may reflect the risk of a cell with a mutant genotype to undergo malignant transformation, rather than identifying the genotype of a specific heritable cancer syndrome. To test this concept, the occurrence of *in vitro* tetraploidy should be evaluated in skin and other tissues from subjects with other heritable cancer syndromes and from cancer prone families.

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